Translational Oncology

www.transonc.com

Volume 3 Number 1 February 2010 pp. 43–49 43

# Inhibition of Thromboxane Synthase Activity Improves Glioblastoma Response to Alkylation Chemotherapy[1]

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## Abstract

Thromboxane synthase (TXSA), an enzyme of the arachidonic acid metabolism, is upregulated in human glial tumors and is involved in glioma progression. Here, we analyzed the *in vitro* and *in vivo* effects of pharmacological inhibition of TXSA activity on human glioblastoma cells. Furegrelate, a specific inhibitor of TXSA, significantly inhibited tumor growth in an orthotopic glioblastoma model by inducing proapoptotic, antiproliferative, and antiangiogenic effects. Inhibition of TXSA induced a proapoptotic disposition of glioma cells and increased the sensitivity to the chemotherapeutic agent 1,3-bis(2-chloroethyl)-1-nitrosourea, significantly prolonging the survival time of intracerebral glioma-bearing mice. Our data demonstrate that the targeted inhibition of TXSA activity improves the efficiency of conventional alkylation chemotherapy *in vivo*. Our study supports the role of TXSA activity for the progression of malignant glioma and the potential utility of its therapeutic modulation for glioma treatment.

*Translational Oncology (2010) 3, 43–49*

## Introduction

Despite recent advances in neurosurgical techniques and the introduction of radiotherapy plus concomitant and adjuvant temozolomide, the prognosis of patients with malignant glioma remains grim [1,2]. The diffusely infiltrative nature of gliomas limits complete surgical removal and is one of the major factors responsible for the almost inevitable tumor recurrence. No anti-invasive therapy is available yet, and current conventional chemotherapeutic approaches have shown only modest efficiency. Toxicity, drug delivery, and the development of drug resistance are the main problems associated with standard chemotherapeutic regimens for malignant brain tumors [3]. Optimization of drug delivery and the development of combinations of compounds that target specific pathobiologic programs of glioma cells are mandatory to overcome these problems [4,5].

Recent data indicated that thromboxane synthase (TXSA), an enzyme of the arachidonic acid pathway, promotes tumor growth and is involved in tumor invasion and metastasis in a variety of cancer types [6–9]. Thromboxane synthase mediates the conversion of the prostaglandin endoperoxide into thromboxane A2 (TXA-2), which is one of the downstream metabolites of cyclooxygenase. Originally, TXA-2 was described for its physiological function as a promoter of platelet aggregation, vasoconstriction, and bronchoconstriction [10]. However, TXSA is highly overexpressed in human gliomas, and expression levels correlate with the grade of malignancy [11,12]. TXSA is involved in the regulation of the migratory phenotype of human glioma cells and provides a potential target for an anti-invasive and proapoptotic strategy [12–14]. The expression of TXSA is coregulated by the tumor suppressor gene p53 and the proto-oncogenic factor ets-1 in an antagonistic fashion [7]. Thus, the development of an apoptosis-resistant and highly invasive glioma phenotype may be due to the loss of p53-mediated negative control over the ets-1–

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[1]This work was supported by the Boston Neurosurgical Foundation and by a grant from the German Research Foundation to N.O.S.

Received 19 August 2009; Revised 23 October 2009; Accepted 28 October 2009



1944-7124 DOI 10.1593/tlo.09238

dependent transcription of the TXSA gene. TXSA-specific inhibitors have been shown to block the migration of human glioma cells *in vitro* and render migration arrested human glioma cell lines highly susceptive to apoptosis-inducing agents [14] and γ-radiation [15]. Therefore, inhibition of TXSA activity is an attractive target for the treatment of human glioblastoma and has the potential to increase the efficiency of conventional chemotherapeutic strategies.

In this study, we investigated the effects of the specific TXSA inhibitor furegrelate on glioma cell sensitivity to the chemotherapeutic agent 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and its therapeutic effects in an intracerebral glioblastoma model. Our results demonstrate that the pharmacological inhibition of TXSA activity inhibits glioblastoma growth by proapoptotic, antiproliferative, and antiangiogenic effects and significantly enhances the survival promoting effects of a systemic BCNU therapy.

## Materials and Methods

### *Cell Culture*

The human glioblastoma cell line U87 (American Type Culture Collection, Manassas, VA) was cultured in α-minimum essential medium (GIBCO BRL, Grand Island, NY) supplemented with 2 mM L-glutamine, 2 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml fungizone, and 10% fetal bovine serum (GIBCO). Cells were maintained in T-75 tissue culture flasks in 5% $CO_2$/95% air at 37°C in a humidified incubator and routinely passaged at subconfluency. For the intracranial implantation experiments, U87 cells were dispersed with a 0.05% solution of trypsin/EDTA (GIBCO), washed with phosphate-buffered saline (PBS), and adjusted to a final concentration of $5 \times 10^4$ cells/10 μl in PBS. 1,3-Bis(2-chloroethyl)-1-nitrosourea (Bristol, Munich, Germany) and the chemically stable and water-soluble specific inhibitor of TXSA, furegrelate (sodium 5-[3′-pyridinylmethyl] benzofuran-2-carboxylic acid; Biomol, Hamburg, PA) were prepared and stored according to the manufacturer's instructions.

### *Cell Growth Assay*

The human glioblastoma cell line U87 was seeded at 2000 cells per well in a 96-well plate and incubated for 30 minutes on ice to allow attachment [11]. The specific TXSA inhibitor furegrelate was added daily, and replicate wells were fixed daily in 1% glutaraldehyde (Serva Electrophoresis GmbH, Heidelberg, Germany). Cells were stained with 50 μl of crystal violet for 1 hour. Cell number was determined in triplicate by absorbance measurements at 540 nm of destained nuclei after 10% SDS treatment.

### *Colony Formation Assay*

The human glioblastoma cell line U87 was seeded in 10-cm culture dishes and maintained until subconfluence. The cultures were treated with the TXSA inhibitor furegrelate and BCNU at doses indicated in the figures. After 24 hours of incubation, cells were trypsinized, washed in PBS, and counted. A single-cell suspension was plated in minimum essential medium containing 10% fetal calf serum into six-well plates at a density of $5 \times 10^5$ cells per well. The cultures were maintained for two weeks, and then the cells were fixed in 1% glutaraldehyde and stained using crystal violet. The number of colonies was counted, and the surviving fraction was calculated as the percentage of colonies of the untreated control.

### *Cell Death Assay*

Apoptosis and necrotic cell death were analyzed using the cell death detection ELISA$^{Plus}$ kit (Roche, Mannheim, Germany) as previously described [14]. In this assay, the intracellular enrichment of mononucleosomes and oligonucleosomes, which occur after induction of endogenous endonucleases, is because, in apoptosis, DNA degradation occurs several hours before plasma membrane breakdown. In contrast, necrotic cell death results in the early release of fragmented DNA into the culture supernatant. Briefly, $2 \times 10^4$ U87 cells were seeded into a 96-well plate and allowed to adhere for 4 hours before treatment. Cells were washed, and the medium was replaced with serum-free medium, after which furegrelate, BCNU, or combinations thereof were added at various concentrations. After 48 hours, the supernatant was aspirated, cells were lysed, and apoptosis as well as necrosis was determined following the manufacturer's instructions. A specific enrichment factor of mononucleosomes and oligonucleosomes released into the cytoplasm was calculated by the absorbance of the treated sample divided by the absorbance of the corresponding untreated control. Values were assessed in triplicate and expressed as mean ± SD.

### In Vivo *Studies*

All animal work was carried out in accordance with federal, local and institutional guidelines. Orthotopic human glioblastoma xenografts were established in 4- to 6-week-old male Swiss nude mice (Taconic, Germantown, NY) as previously described [16]. Mice were anesthetized (100 mg/kg ketamine and 5 mg/kg xylazine) and received a stereotactically guided injection of $5 \times 10^4$ human U87 glioma cells into the left basal ganglia (2 mm lateral and 1 mm anterior to bregma, at a 3-mm depth from the skull surface) after a small craniectomy. Eight days after tumor cell injection, 5 to 10 mice were randomized for the treatment and control groups. Each experiment was performed in duplicate. In the first experiment (end point study), Alzet osmotic minipumps (Durect Corporation, Cupertino, CA) designed to deliver 0.25 μl/h for 4 weeks were used for intratumoral drug delivery as previously described [16]. The pumps were filled with furegrelate to deliver 0.5 or 2 mg/kg per day, or PBS for the control animals, and were connected to a brain infusion cannula through a catheter tube according to the manufacturer's instructions (Brain Infusion Kit, Durect). To establish a constant flow before implantation, the filled pumps were incubated overnight in sterile PBS at 37°C. Eight days after injection of U87 glioma cells, the pump reservoirs were placed subcutaneously in the left flank of the anesthetized mice. The brain infusion cannula was tunneled subcutaneously and fixed to the skull with surgical glue at the initial tumor cell injection site resulting in an intratumoral localization of the cannula tip (~2-2.5 mm in depth). Closure of the incisions was performed by suture. All groups were killed by $CO_2$ inhalation 21 days after treatment was initiated. Brains were removed, embedded in OCT, and stored at −80°C until further processed for histologic analysis. In the second experiment (survival study), the effects of the local microinfusion of furegrelate alone and in combination with systemic administration of BCNU on the survival of U87 human glioblastoma-bearing nude mice were assessed. Implantation of osmotic minipumps 8 days after tumor cell injections was performed as described previously. Pumps were filled either with PBS or with furegrelate to deliver 2 mg/kg per day in PBS solution. Systemic BCNU treatment [17] was performed by single daily intraperitoneal injections (i.p.; 15 mg/kg per day) for 5 days starting day 10 after tumor cell injection. In this experiment, animals were killed at the onset of neurologic signs or any type of distress.

### *Immunohistochemical Analyses*

Frozen brains were cut in serial 10-µm sections and stained with hematoxylin and eosin for histologic evaluation. Tumor volumes were calculated by the ellipsoid formula [18]. Immunohistochemistry was carried out using standard techniques with the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Primary antibodies included anticleaved caspase 3 (1:100; Cell Signaling Technology, Beverly, MA) for the detection of apoptosis, anti-CD31 (1:100; BD Biosciences Pharmingen, San Jose, CA) for blood vessels and anti-Ki67 nuclear antigen (1:100; DAKO, Carpinteria, CA) for detection of cell proliferation. Sections were counterstained with hematoxylin, and negative control slides were obtained by omitting the primary antibody. The apoptosis and proliferation index were quantified by counting the number of positively stained cells of 100 nuclei in five randomly chosen high-power fields. The intratumoral microvessel density was assessed as described by Leon et al. [19]. For the identification of hemosiderin deposits as a sign for microhemorrhages, we used the Prussion-Blue reaction with a neutral red counterstain.

### *Statistics*

All values were calculated as mean ± SD or expressed as percentage of control ± SD. Significant differences between tumor volumes, microvessel density, proliferation, and apoptosis index were determined using the Mann-Whitney rank sum test (MW test) or the unpaired *t* test. Kaplan-Meier survival curves were statistically analyzed using the Cox regression analysis.

## Results

### *Proapoptotic Synergism of the Specific TXSA Inhibitor Furegrelate with BCNU on Human U87 Glioblastoma Cells*

We have previously shown that human glioblastoma including the human glioblastoma cell line U87 expresses both TXSA and the thromboxane receptor [12,15]. Treatment of U87 glioma cells with furegrelate leads to migration arrest and induction of apoptosis. Further, pretreatment with furegrelate sensitized U87 cells to radiation-induced cell death [15]. To test whether TXSA inhibitors inhibit the growth of U87 cells, *in vitro* growth curve experiments using increasing concentrations of furegrelate were performed. This demonstrated that single doses of furegrelate as low as 0.1 mg/ml added to the culture medium inhibited cell growth (Figure 1*A*). Confirming our previous findings, furegrelate resulted in a significant histone-complexed DNA fragmentation of U87 cells in an apoptosis assay 48 hours after

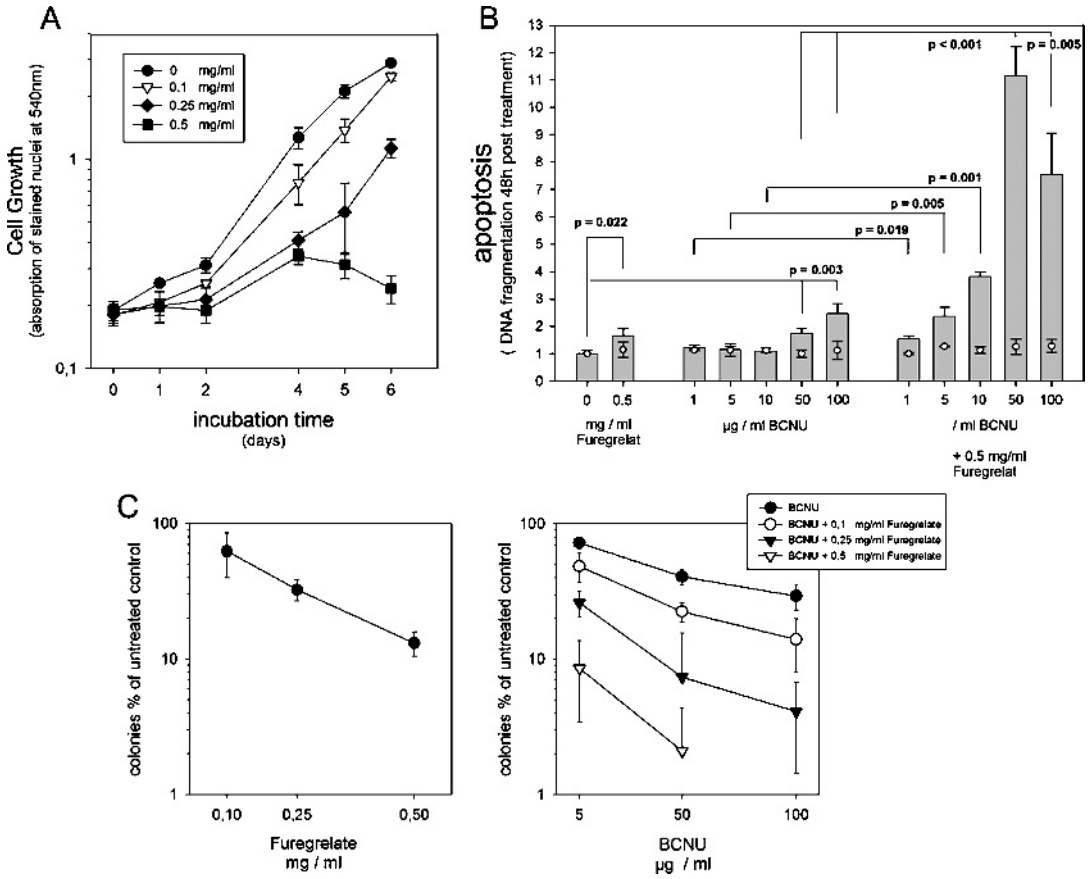


**Figure 1.** *In vitro* effects of the specific TXSA inhibitor furegrelate on the human U87 glioblastoma cell line. (A) Increasing concentrations of furegrelate reduced the cell growth of U87. (B) Cotreatment of U87 with furegrelate and BCNU leads to a robust synergistic apoptotic effect *in vitro* indicating a sensitizing effect of furegrelate. Tumor cells were incubated with indicated concentrations and combinations of furegrelate and/or BCNU for 48 hours. DNA fragmentation was determined in cellular lysates (bars) and culture supernatant (o). No significant amounts of DNA fragments could be detected in the cell culture supernatant indicating that the effects were a consequence of apoptotic cell death and not of cellular necrosis. All values shown are mean ± SD. (C) Cotreatment with furegrelate and BCNU lead to a synergistic decrease in the surviving fraction of U87 tumor cells as determined by a colony formation assay. Values were calculated as mean ± SD of triplicate determinations.

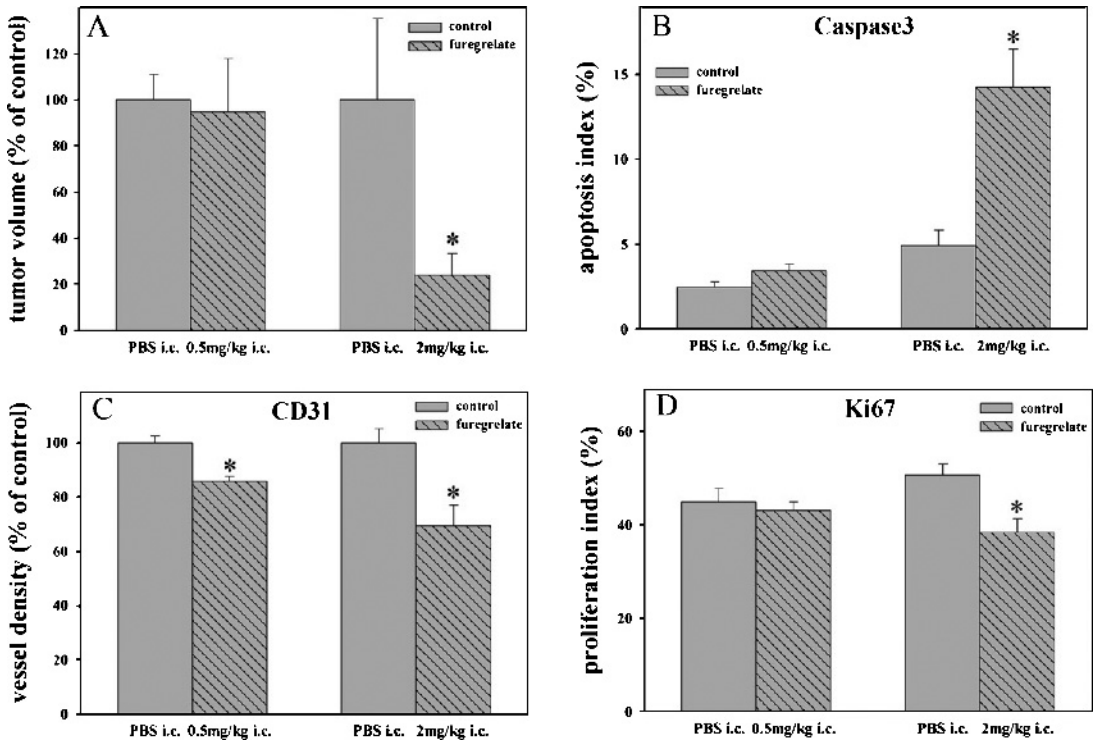


**Figure 2.** The local intracerebral infusion (i.c.) of 2 mg/kg per day of furegrelate is more effective than 0.5 mg/kg per day on established human U87 glioblastoma xenografts in nude mice. (A) The local delivery of 2 mg/kg per day of furegrelate reduced the tumor growth of U87 human glioblastoma xenografts by 73.1% (*$P < .05$) as assessed by histologic volume measurement 21 days after treatment was initiated. Treatment with 2 mg/kg per day of furegrelate induced (B) a significantly increased apoptosis index (2.9-fold, *$P < .005$). Furthermore, treated tumors displayed (C) a significantly reduced intratumoral microvessel density (30.4%, *$P < .05$) and (D) a significant reduction of tumor cell proliferation (24.1%, *$P < .01$).

treatment. BCNU treatment at concentrations of 50 and 100 μg/ml also induced apoptosis. However, cotreatment of 0.5 mg/ml furegrelate and increasing doses of BCNU demonstrated significantly increased intracellular DNA fragmentation over the single-agent treatments (Figure 1*B*). To demonstrate a synergistic effect of furegrelate and BCNU, a colony formation assay was used. This showed that increasing concentrations of furegrelate and BCNU lead to a dose-dependent decrease of the clonogenic surviving fraction of U87 cells (Figure 1*C*).

### *The TXSA Inhibitor Furegrelate Inhibits Intracerebral Glioblastoma Growth by Proapoptotic, Antiproliferative, and Antiangiogenic Effects* In Vivo

We next determined the *in vivo* effects of furegrelate on the intracerebral growth of established human U87 glioblastoma xenografts. To gain insight into the mechanism of action of TXSA inhibition, all animals were killed 21 days after initiation of treatment, and the brains were processed for immunohistochemical analyses. Therapeutic local intracerebral microinfusions were established by implantation of osmotic minipumps at the tumor site 8 days after glioma cell injection. The direct intracerebral release of a daily total dose of 2 mg/kg of the specific TXSA inhibitor furegrelate resulted in a significant tumor growth inhibition when compared with the control group receiving intralesional infusions of PBS (73.1%, $P < .05$; Figures 2*A* and 3, *A* and *A′*). This antitumor effect was mediated by a significant increase in the apoptosis index (2.9-fold, $P < .005$; Figures 2*B* and 3, *B* and *B′*) and a significant reduction of the intratumoral microvessel density (30.4%, $P < .05$; Figures 2*C* and 3, *C* and *C′*) and tumor cell proliferation (24.1%, $P < .01$; Figures 2*D* and 3, *D* and *D′*). These proapoptotic, antiangiogenic, and antiproliferative effects of TXSA inhibition were dose-dependent because no significant antitumor effects were observed at 0.5 mg/kg per day of furegrelate (Figure 2, *A–D*). However, at this dose, the intratumoral blood vessel density was still slightly reduced by 14% ($P < .05$; Figure 2*C*) when compared with the control group, indicating activity of furegrelate on inhibition of TXSA, although not sufficient to elicit a significant antitumor effect. Local intracerebral delivery of furegrelate seemed safe because animals from all groups did not show any significant weight loss or signs of adverse effects due to the treatment. Histologic screening of the brain sections did not reveal any signs for intratumoral hemosiderin deposits, indicating that the antiangiogenic effects did not induce any intracerebral hemorrhage. Only in one control and in one furegrelate-treated animal that minor hemosiderin deposits were identified at distance from the tumor and the cannula site of the pump. All pump reservoirs displayed a decreased volume at the end of the experiments, indicating that furegrelate was delivered as expected.

### *Local Therapy with Furegrelate in Combination with Systemic BCNU Therapy Improves Survival in an Intracerebral Glioblastoma Model*

We next asked whether the inhibition of TXSA activity by local intracerebral microinfusion of furegrelate results in an increased survival time of nude mice with established orthotopic human U87 glioblastoma xenografts. Furthermore, we were interested if the proapoptotic

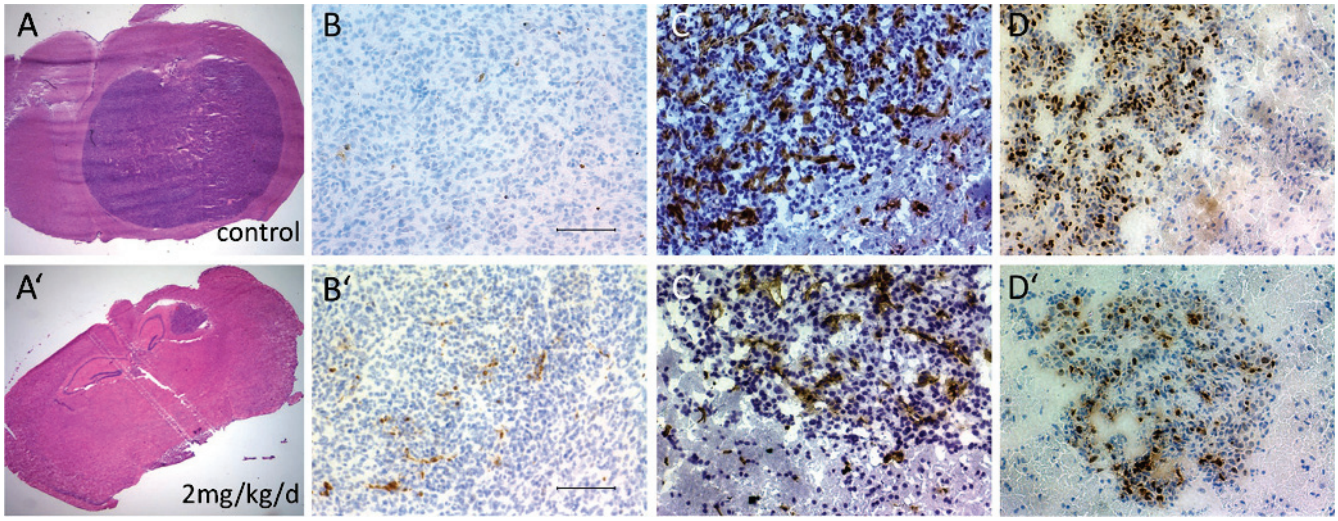


**Figure 3.** Histologic examination after 21 days of treatment with the local intracerebral microinfusion of 2 mg/kg per day of furegrelate. Representative examples of U87-derived tumors grown in the brain of animals that received PBS i.c. (A–D) or 2 mg/kg per day of furegrelate locally (A′–D′). (A, A′) Frozen sections stained with hematoxylin and eosin. (B, B′) Apoptotic tumor cells as assessed by immunoreactivity for cleaved caspase 3. (C, C′) Microvessel density as assessed by immunoreactivity for the endothelial cell marker CD31. (D, D′) Proliferating tumor cells as assessed by immunoreactivity for Ki67. Scale bar, 100 $\mu$m.

disposition due to inhibition of TXSA activity would enhance the therapeutic benefits of systemically administered BCNU chemotherapy. Osmotic minipumps were implanted at the tumor site 8 days after tumor cell injection. The pump reservoirs were filled either with PBS for the control group or with furegrelate at a total daily dosage of 2 mg/kg. BCNU-treated animals received a single daily i.p. injection of 15 mg/kg BCNU for 5 days starting at day 10 after tumor cell injection. The antitumor effects of furegrelate at 2 mg/kg per day did not translate into a significant survival benefit compared with PBS-treated animals ($P$ = .211; Figure 4). Whereas most animals in the control group had died by day 30, three animals of the furegrelate-treated group lived longer than day 36, which was the maximum duration of furegrelate delivery by the osmotic minipumps. The dosage of 15 mg/kg per day of BCNU significantly increased the mean survival time by 9 days compared with the control group ($P$ < .0001). However, the pharmacological inhibition of TXSA by furegrelate (2 mg/kg per day) followed by a systemic BCNU therapy significantly prolonged the survival time ($P$ < .0001; Figure 4). The median survival time was increased by 16 days compared with the controls in this highly aggressive orthotopic glioblastoma model. We observed no clinical apparent adverse effects and no significant weight loss in the treatment groups.

## Discussion

The current standard of care for patients with malignant glioma consists of surgical tumor resection followed by a combination of radiotherapy

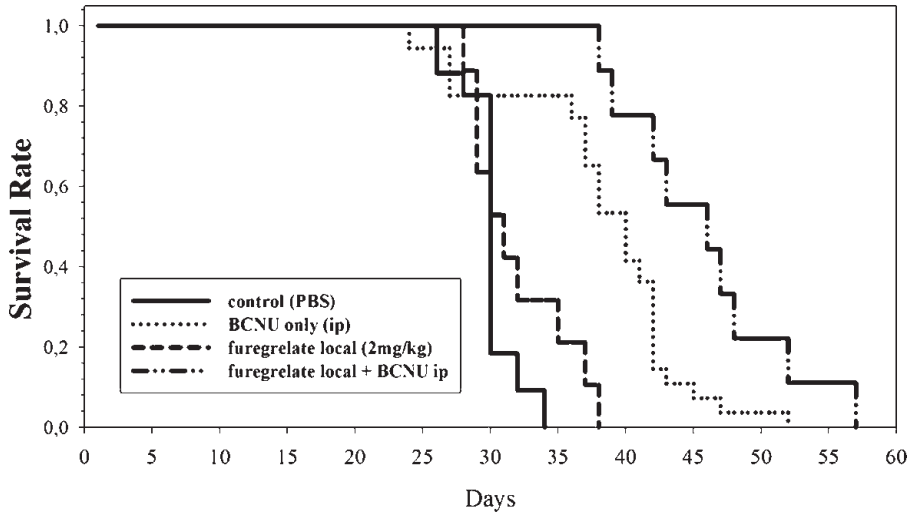


**Figure 4.** Kaplan-Meier survival curve of human U87 glioblastoma-bearing nude mice. The local intracerebral microinfusion (i.c.) of furegrelate at 2 mg/kg per day from day 8 to 36 in combination with a BCNU chemotherapy administered i.p. at 15 mg/kg per day from day 10 to 14 further increased the survival benefits of BCNU therapy ($P$ < .0001).

with temozolomide [1]. Despite a statistically relevant benefit in overall survival for patients with a newly diagnosed glioblastoma, this improvement remains modest from a clinical viewpoint. Tumor recurrence is still inevitable, and further meaningful therapeutic options are still unresolved. BCNU has been used as an adjuvant chemotherapeutic drug in the treatment of malignant glioma for decades [20]. Recently, the implantation of biodegradable BCNU-releasing wafers after surgical removal of a tumor mass has been demonstrated to offer a survival benefit to patients with primary and recurrent glioblastoma [21,22]. It is the first clinically approved local-regional chemotherapy for malignant glioma patients. Nevertheless, the development of combinations of compounds that target specific pathobiologic programs of glioma cells and the potential to enhance the effectiveness of current chemotherapeutic strategies are clearly needed [5,23].

The overexpression of TXSA is associated with tumor progression and metastasis in a variety of cancer types [8,12,24–26]. Cancer-promoting effects of TXSA activity seem to include the augmentation of tumor angiogenesis and tumor cell invasion [6,12,25,27]. However, the *in vivo* effects of TXSA inhibition have been hardly investigated in orthotopic cancer models. In this study, we demonstrated that the pharmacological inhibition of TXSA activity by furegrelate inhibits intracerebral glioblastoma growth. This tumor inhibition was mediated by proapoptotic, antiproliferative, and antiangiogenic effects. Our findings are supported by a previous study demonstrating that the experimental overexpression of TXSA promoted tumor growth in an adenocarcinoma mouse model by enhancing angiogenesis [6]. Intratumoral endothelial cells of malignant gliomas are known to express high levels of TXSA [12,28]. TXA-2, the active metabolite of TXSA activity, is involved in the angiogenic process by stimulating endothelial cell migration and vascular smooth muscle cell proliferation [10,27,28]. Furthermore, TXA-2 synthesis in endothelial cells is stimulated by fibroblast growth factor-2 and vascular endothelial growth factor, which are highly expressed in malignant glioma and are essential key players of glioma progression [27,29]. Our data confirmed the relevance of TXSA activity for glioma angiogenesis *in vivo*. Pharmacological inhibition of its activity led to a significant reduction of intratumoral microvessel density. We observed an increased glioma cell apoptosis and a reduced tumor proliferation rate, which may be in part due to the antiangiogenic effects of TXSA inhibition. However, our *in vitro* data demonstrated that furegrelate exerts potent direct antitumor effects by impairing cell growth, inducing apoptosis, and decreasing clonogenic cell survival of human U87 glioblastoma cells.

TXSA overexpression in glioma cells was initially identified in a human glioma cell line selected for a highly migratory phenotype by using differential messenger RNA expression analysis [13]. We have previously demonstrated that the level of TXSA activity correlates with the *in vitro* migration rate of a variety of human glioma cell lines and that pharmacological inhibition of this enzyme blocks tumor cell motility [12]. Remarkably, this increased the *in vitro* sensitivity to apoptosis induced by etoposide, camptothecin, or BCNU [14]. A similar sensitizing effect of TXSA inhibition was recently shown for bladder cancer cell lines in response to cisplatin and paclitaxel [9], but the relevance of the increased tumor cell chemosensitivity has never been investigated *in vivo*. Here, we expanded these findings and demonstrated that furegrelate induced a proapoptotic disposition of human glioblastoma cells and increased the sensitivity to the chemotherapeutic drug BCNU *in vivo*, which significantly prolonged the survival time of intracerebral glioma-bearing mice.

We have previously shown that inhibition of TXSA by furegrelate also sensitized experimental glioma to radiation therapy [15]. γ-Radiation alone led to an induction of endogenous thromboxane synthesis, implicating TXSA as a survival factor. Metabolites of the arachidonic acid pathway influence a multitude of cellular properties [30]. Inhibition of TXSA activity may lead to an accumulation of further upstream metabolites possibly activating alternative pathways, which may be responsible for the failure of pharmacological TXSA inhibition alone to prolong the survival of intracerebral glioma-bearing mice despite its potent antitumor effects in our end point study.

It seems reasonable to speculate that therapeutic inhibition of TXSA activity has the clinical potential to inhibit the invasive phenotype of glioma cells. Current experimental glioblastoma models do not reflect the full invasive phenotype of human tumors. However, recent findings indicated that intracerebral xenografts of glioblastoma-derived stem cell lines display a highly invasive tumor growth and may be ideal models to evaluate anti-invasive strategies in future studies [31]. Among the compounds tested for modulating the metabolic pathway of arachidonic acid, furegrelate displays the most potent antitumor effects on human glioma cells [11,32]. TXSA inhibitors have undergone clinical testing for modulating platelet aggregation or vasoconstriction in a variety of diseases [10]. Human clinical studies with furegrelate indicated that it is a well-tolerated effective inhibitor of thromboxane synthesis when administered chronically [33,34]. Despite its known modulation of platelet aggregation and its antiangiogenic effects, we did not observe any intracerebral hemorrhage in our study. This is the first study demonstrating that the pharmacological inhibition of TXSA sensitizes glioma cells to alkylation chemotherapy *in vivo*. Our data warrant further investigation of its value as an adjuvant therapeutic strategy for glioma treatment. Taken together, targeted inhibition of TXSA activity may have the potential to improve the efficiency of conventional chemotherapy and radiotherapy.

## Acknowledgments

The authors thank Elizabeth Allred for help with the statistical analysis.

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